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Zoological Research

Phylogenetic Relationships of Monal Pheasants *Lophophorus* Inferred from Sequences of Mitochondrial Cytochrome *b* Gene

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Abstract: The phylogeny of the monal pheasants (*Lophophorus*) and their relationships to some species of the genera *Tragopan*, *Pucrasia* and *Ithaginis* were studied by comparing mitochondrial cytochrome *b* (cyt *b*) nucleotide sequences. The molecular phylogenetic trees show that: ①the genus *Tragopan* and the genus *Pucrasia* share a common ancestor which is the sister taxon of the ancestor of the genus *Lophophorus*; ②the genus *Lophophorus* had evolved into two branches: One was the Sclater's Monal; the other included the Chinese Monal and the Himalayan Monal. Considering its molecular phylogeny, distribution patterns and morphological evidences, the genus *Lophophorus* might originate in the Hengduan mountains region of southwestern China.

Key words: *Lophophorus*; Pheasant; Phylogeny; Cyt *b* gene

基于线粒体细胞色素 *b* 基因序列的虹雉属鸟类的系统发育关系

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摘要: 通过雉科虹雉属(*Lophophorus*)、角雉属(*Tragopan*)、勺鸡属(*Pucrasia*)和血雉属(*Ithaginis*)7种鸟类的细胞色素 *b* (cyt *b*) 基因序列比较, 构建的虹雉属及其近缘属的分子系统树表明: ①3种虹雉构成一个单系群(monophyletic group), 虹雉属与角雉属、勺鸡属构成一个单系群; ②虹雉属内分为白尾梢虹雉, 以及棕尾虹雉和绿尾虹雉两个演化枝。综合分子系统学、地理分布格局和形态学的证据, 推测虹雉属鸟类起源于中国的横断山脉, 其中繁衍生活在原地的一枝演化为白尾梢虹雉; 另一枝则分别进入喜马拉雅山区(西)和中国西南部(东), 向西的演化为棕尾虹雉, 向东的则为绿尾虹雉。

关键词: 虹雉属; 雉类; 系统发育; 细胞色素 *b* 基因
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The monals (*Lophophorus*) are large montane pheasants in which the sexes are highly dimorphic. Currently, three species of the genus *Lophophorus* have been identified, namely, the Himalayan Monal (*L. impejanus*), the Chinese Monal (*L. lhuysii*) and the Sclater's Monal (*L. sclateri*) (Delacour, 1977; Cheng Tso-hsin, 1978; Johnsgard, 1999). Apart from the Himalayan Monal that occurs in the Hi-

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malayan regions, most of the other monals distribute in southwestern China including Tibet, Yunnan, Sichuan, Qinghai and Gansu. The Hengduan Mt. is the core area for the monals. There are sympatric distributional sites of the three species in this region, such as Chayu county of Tibet. As the wild populations of monals are on the process of decline due to habitat destruction and human disturbance, all species of the genus have been listed as the national first-grade protected wildlife of China since 1988 (Zheng & Wang, 1998).

Although there were some intensive studies on the ecology of Chinese Monal in the past decades (He & Lu, 1985; 1986), the evolutionary history and phylogeny of the genus *Lophophorus* remain unclear. Based on comparison of the morphological characteristics, Delacour (1977) concluded that the genus *Lophophorus* was an ancient and long-established genus, with no apparent phyletic links to the other genera. Johnsgard (1999) suggested that, among the lineage of tragopans and their allies, the genus of *Lophophorus* was one of the earliest branches evolved, while the other branch firstly evolved into the Koklass Pheasant (*Pucrasia macrolopha*), then the Blood Pheasant (*Ithaginis cruentus*) and tragopans (Fig.1a). By examination of amino acid sequences of the mitochondrial cytochrome *b* (cyt *b*) from the Himalayan Monal and some other pheasants, Kimball et al (1999) agreed with the points proposed by Johnsgard (1999), but they argued that the Blood Pheasant was the basal branch of the lineage and the Himalayan Monal, Koklass Pheasant and tragopans (Temminck's Tragopan, *Tragopan temminckii*) form the other branch (Fig.1b). So far, there is no research about the phylogenetic relationships among the three species in the genus *Lophophorus*, except for Johnsgard's (1999) suggestion that they might form a geographical replacement series and the Chinese Monal was the basal branch followed by the divergence between the Himalayan Monal and the Sclater's Monal (Fig.1a).

In the avian family Phasianidae, mitochondrial DNA (mtDNA) genes, especially cyt *b*, have been sequenced to study the phylogenies of many genera (Fumihito et al, 1995; Randi et al, 2000). In this study, the cyt *b* sequences of all three species of *Lophophorus* were obtained and compared with that of other species in the close related genera. The aims of phylogenetic analyses of these data are: ①to examine the phylogenetic position of the genus *Lophophorus*; ②to study the phylogenetic relationships among the three monal species; ③to combine the molecular data, distribution patterns and morphological traits to infer the evolution process of the genus *Lophophorus*.

1 Materials and Methods

1.1 Sampling, DNA extraction, PCR amplification and sequencing

Total DNA was extracted from the molted feathers

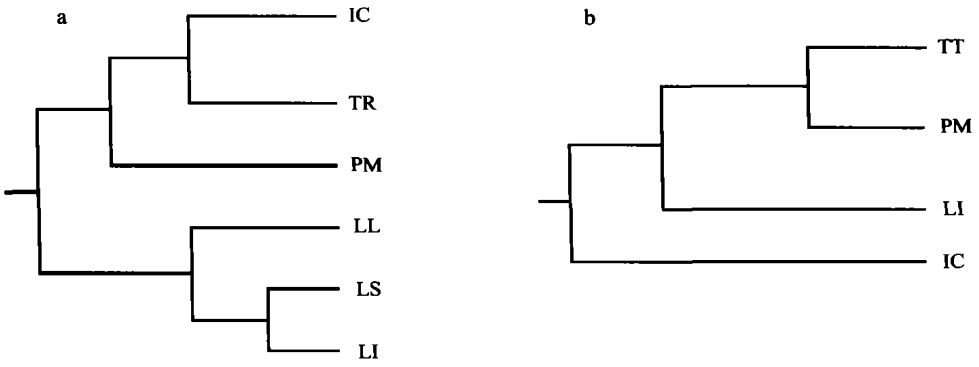

Fig.1 Phylogenetic trees of the genus *Lophophorus* and related genera

a. Modified form Johnsgard (1999); b. Modified from Kimball et al (1999).

IC: *Ithaginis cruentus*; TR: *Tragopan*; PM: *Pucrasia macrolopha*; LL: *L. lhuysii*; LS: *L. sclateri*; LI: *L. impejanus*; TT: *Tragopan temminckii*.

of the Chinese Monal, which bred in captivity in Beijing Zoo with well-documented lineage, and the Sclater's Monal caught from wild population in Yunnan and reared in the Beijing Breeding Center for Endangered Animals. In the sampling process, plastic gloves were used to avoid contamination of the samples. The feathers were then preserved at −80 ℃ to prevent DNA from further degradation.

The roots of feathers were rinsed with distilled water. 3 mm-long calami were scissored and cut into pieces with sterile scissors. DNA extraction protocol was modified from Taberlet & Bouvet (1991). The concentration of DNA dissolved in TE (10 mmol/L Tris-HCl, pH 8.0, 1 mmol/L EDTA) was determined with the spectrophotometer (DU-640, BECKMAN, Germany). DNA samples were stored in freeze at −20 ℃.

The cyt *b* gene of the Chinese Monal and Sclater's Monal were PCR amplified using a set of five primers (L14731, L14788, L15164, L15400, H16065, see Table 1). Amplifications were carried out with 25 uL reaction buffer containing 100 ng DNA template, 0.5 μmol each primer, 1 U Taq DNA polymerase (Takara, Japan), 2.5 mmol/L $MgCl_2$ (Takara), 0.2 mmol/L dNTP mixture (Takara) and 1 × PCR Buffer (Takara). It was performed on a thermocycler following the standard steps: 94 ℃, denatured for 5 min; then 40 cycles (1 min at 94 ℃, 1 min at the corresponding *Tm* of each pair of primers, 1.5 min at 72 ℃); 72 ℃ extension for 10 min. After PCR products were examined by agarose electrophoresis (PROMEGA, USA), the target bands were cut, purified by the Gel Extraction Mini Kit (Watson Biotech, China) and quantified.

DNA sequencing reactions were performed using ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems Inc., USA). DNA sequences were determined by ABI 310 Collection Software (v2.0) and the Sequence Analysis Software 3.4.1. The primers used for both the amplification and sequencing are listed in Table 1.

1.2 Phylogenetic analyses

The whole cyt *b* sequences of the Chinese Monal and Sclater's Monal were obtained by overlapping the partial sequences of the cyt *b* with the software SeqEdit (Applied Biosystems Inc., USA). Other species used in the phylogenetic analyses were downloaded from the GenBank (Table 2). As the cyt *b* sequences are uniform in length, the alignment was straightforward. In this study, Blue-breasted Quail (*Coturnix chinensis*) was used as the outgroup.

The genetic distance, nucleotide compositions and variability at different positions were estimated using the software Mega2 (Kumar et al, 2001). The PAUP4.0 (Swofford, 1998) were used to construct: ①maximum parsimony (MP) tree; ②maximum likelihood (ML) tree by heuristic search. The best-fit ML model of DNA substitution, GTR + I + G, was selected by Modeltest 3.06 (Posada & Crandall, 1998) and implemented in the ML analyses. We assessed the reliability of the clades in phylogenetic trees by bootstrap percentage (BP; Felsenstein, 1985) computed using 1 000 replicates with 10 random additional sequencing replicates for each bootstrap replicate.

Bayes tree was computed by the execution of

Table 1 Amplification and sequencing primers for cyt *b* genes of *Lophophorus*

Name*	Sequence (5′→3′)	Source
L14731	ATCGCCTCCCACCT (AG) AT (CG) GA	Kimball et al, 1999
L14788	TGCCAACCTTCATCTTATTAT	This study
L15164	GCAAACGGCGCCTCATTCTT	Kimball et al, 1999
L15400	AGGGTTGGGTTGTCGACTGA	Kimball et al, 1999
L15662	CTAGGCGACCCAGAAAACTT	Kimball et al, 1999
H16065	TTCAGTTTTTGGTTTACAAGAC	Kimball et al, 1999

* Name indicates light (L) or heavy (H) strand and the position of the 3′ end of the oligonucleotide numbered according to the chicken mitochondrion (Desjardins & Morais, 1990).

Table 2 Species examined and source of sequence data

Species	Common name	Accession No.
Lophophorus impejanus	Himalayan Monal	AF028796
Lophophorus lhuysii	Chinese Monal	AY265309
Lophophorus sclateri	Sclater's Monal	AY265310
Tragopan caboti	Cabot's Tragopan	AF534554
Tragopan blythii	Blyth's Tragopan	AF200722
Pucrasia macrolopha	Koklass Pheasant	AF028800
Ithaginis cruentus	Blood Pheasant	AF068193
Coturnix chinensis	Blue-breasted Quail	NC004575

MCMC (Markov Chain Monte Carlo) in the software. MrBayes (Huelsenbeck & Ronquist) and its reliability were assessed by the posterior probability (PP).

2 Results

2.1 Molecular evolution of cyt *b* sequences

Nucleotide frequencies were not significantly different among species, and thus the Tamura-Nei model (Gamma) is an appropriate estimator of genetic distance ($\alpha = 1.92$, computed by Modeltest 3.06). The average observed genetic distances (D) (Table 3) of cyt *b* sequences among the three species of monals is 0.061 ± 0.007. Interspecific values of D in the genus *Lophophorus* are 0.064 ± 0.009 (*lhuysii-sclateri*), 0.058 ± 0.008 (*impejanus-sclateri*) and 0.060 ± 0.008 (*impejanus- lhuysii*) respectively. The third position of the codon is the most variable one of the three positions, for example, 254 of 333 variable sites are in the third position. Most parsimony informative sites are also in the third positions of the codon.

2.2 Molecular phylogenetic trees

Based on the cyt *b* sequences, the MP, ML and Bayes trees of the genus *Lophophorus* plus species from other genera were constructed. The MP and ML tree had the same topology (Fig.2).

3 Discussion

3.1 Phylogenetic position of the genus *Lophophorus*

The genus *Lophophorus* was clearly monophyletic despite different phylogenetic methods used in the analyses and the clade was well resolved with BP = 99% in both the MP and ML tree (Fig.2a) and PP = 100% in the Bayes tree (Fig.2b). Based on the molecular data, our results support the point proposed by Johnsgard (1999) that the genus *Lophophorus* included three species, the Himalayan Monal, the Chinese Monal and the Sclater's Monal.

Table 3 Genetic distance (TN, below the diagonal) with SD (above the diagonal) among the monals and other pheasants

Species	1	2	3	4	5	6	7
1 *Ithaginis cruentus*		[0.017]	[0.015]	[0.014]	[0.014]	[0.018]	[0.016]
2 *Tragopan caboti*	0.183		[0.014]	[0.013]	[0.013]	[0.009]	[0.013]
3 *Lophophorus lhuysii*	0.161	0.135		[0.009]	[0.008]	[0.013]	[0.014]
4 *Lophophorus sclateri*	0.147	0.120	0.064		[0.008]	[0.014]	[0.013]
5 *Lophophorus impejanus*	0.143	0.131	0.060	0.058		[0.014]	[0.013]
6 *Tragopan blythii*	0.192	0.076	0.130	0.142	0.137		[0.014]
7 *Pucrasia macrolopha*	0.169	0.128	0.130	0.123	0.127	0.142	
8 *Coturnix chinensis*	0.195	0.174	0.158	0.162	0.167	0.179	0.168

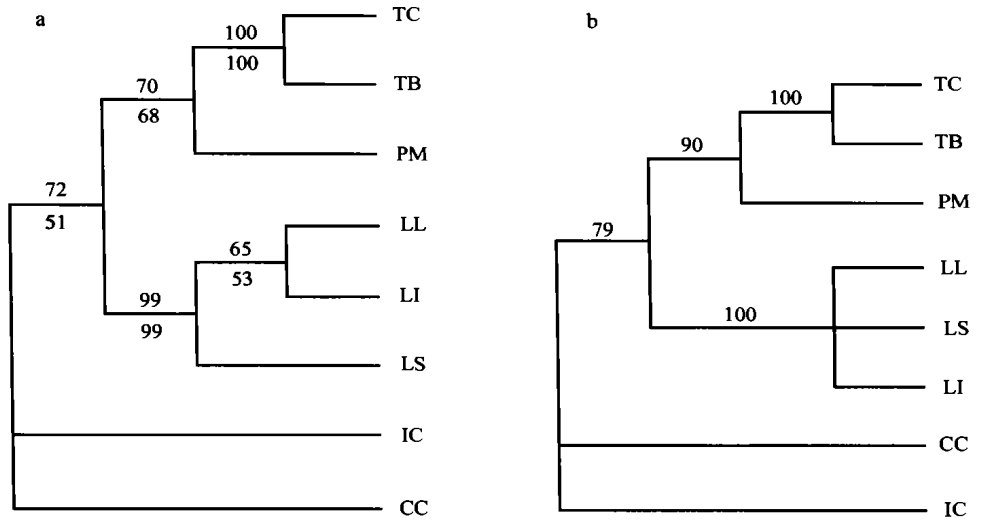

Fig.2 Phylogenetic trees of the monals and other pheasants based on the cyt *b* sequences
a. MP and ML trees (BPs of the MP and ML are above and below the branch respectively); b. Bayes tree with PP.
TC: *Tragopan caboti*; TB: *Tragopan blythii*; PM: *Pucrasia macrolopha*; LL: *L. lhuysii*; LS: *L. sclateri*; LI: *L. impejanus*; IC: *Ithaginis cruentus*; CC: *Coturnix chinensis*.

Although the BPs of the branch composed of the genera *Tragopan* and *Pucrasia* were not very high (≤ 70%) in the MP and ML tree, it was reported that the bootstrap percentage was a conservative estimator (Hills & Bull, 1993; Kimball et al, 1999). Moreover, the PP of Bayes tree was high (90%). Therefore, the two genera should have a common ancestor which is the sister taxon of the ancestor of the genus *Lophophorus* (see Fig.2). Our data support the point that the genera *Tragopan*, *Pucrasia* and *Lophophorus* form a monophyletic group (Fig.1b; Kimball et al, 1999), but don't support the lineage, the tragopans and their allies (compare Fig.1 with Fig.2) because the phylogenetic position of the genus *Ithaginis* is not resolved in the analyses. Both the phylogenetic trees and the genetic distances suggest that the Blood Pheasant is older than other studied pheasants in its evolutionary history. However, whether the *Ithaginis* belongs to the phasianini (Johnsgard, 1999) or belongs to the perdicini (Dyck, 1992), is still uncertain. Therefore, the phylogenetic position of the genus *Ithaginis* needs further research.

3.2 Phylogenetic relationships within the genus *Lophophours*

By the MP and ML analyses, we found that the genus *Lophophorus* had evolved into two branches: One was the Sclater's Monal and the other was composed of the Himalayan Monal and the Chinese Monal. This conclusion was not concordant with the two branches proposed by Johnsgard (1999), i.e. the Himalayan-Sclater's Monal and the Chinese one. There are also several lines of morphological evidence to support our point about the evolutionary pattern of the genus: ① Both the Chinese Monal and the Himalayan Monal have long crest, while the Sclater's Monal has a very short and undiscerned crest; ②The upper tail converts of the former two are blue and green in contrast to the white of the Sclater's Monal (Johnsgard, 1999).

Considering its molecular phylogeny and geographical distribution patterns, we proposed that the monals might originate in the Hengduan mountains region according to the rule that the taxon diversity of the origin center is the richest (Nelson & Platnick, 1984), which is consistent with the point that the major origin center of the pheasants is in the eastern Himalayas and across northern Burma and Yunnan (Johnsgard, 1999; Randi et al, 2000). The hypothetical ancestral populations of the genus could have been located in this area and evolved into two branches with $D = 0.061 \pm 0.007$ between them. If the general molecular clock, 2.0%

average divergence per million years (myr), is taken (Shields & Wilson, 1987), the Sclater's Monal branch should diverge from the ancestor of the monals 3.05 ± 0.35 myr ago. The Scalter's Monal has stayed and lived in the Hengduan mountains region for a long history. But the other branch was progressively fragmented or dispersed, partly due to the great terrain and environment changes in the Hengduan mountains in the Pliocene, which produced the western and the eastern *Lophophorus* lineages that spread towards the Himalayas and southwestern China, respectively. The populations in the west were gradually adapted to the environment and finally evolved into the Himalayan Monal, while the eastern populations evolved into the Chinese Monal. We must admit that the latter branch is low in the BP (< 70%), which maybe result from the short interval between the divergence of the Sclater's Monal (3.05 ± 0.35 myr ago) and the divergence between the Himalayan Monal and Chinese Monal (2.9 ± 0.4 myr ago).

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